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Expression of Active Human Blood Clotting Factor VIII in the Mammary Gland of Transgenic Rabbits

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ABSTRACT

Human clotting factor VIII is probably the largest protein to be expressed to date in the mammary gland of a transgenic animal, and it requires extensive posttranslational modification to achieve full biological activity. The mammary gland specific construct mWAP-hFVIII-MT-I was injected into the pronuclei of rabbit zygotes, and three transgenic offspring were obtained. Founder 385 showed germ-line transmission of a single integrated copy, and a homozygous line was established from this animal. The rhFVIII was transcribed and translated exclusively in the mammary gland. The activity of rhFVIII in the rabbit milk ranged from 5 to 8% of that found in normal human plasma. Results indicate the suitability of the transgenic rabbit mammary gland for rhFVIII production.

INTRODUCTION

HEMOPHILIA A IS AN X-LINKED congenital bleeding disorder affecting approximately 1 in 10,000 males caused by mutations in the gene for blood clotting factor VIII (FVIII) (Kazazian *et al.*, 1995). Recombinant human factor VIII (rhFVIII) is currently produced commercially in cell culture systems, and is widely used for replacement therapy. Unfortunately, the amounts available do not meet the worldwide demand, and the product shows considerable variability in post-translational modification (Garber, 2000).

The technology for using the mammary gland as a bioreactor has been developed to the point that pharmaceuticals derived from the milk of transgenic farm animals are currently in the advanced stages of clinical trials (Dove, 2000). The time required to generate a transgenic animal with high expression levels and to deliver a product to the market are the major drawbacks of large animal transgenic technology. Transgenic rabbits offer an attractive alternative to large dairy animals because of their large litter size and short generation interval (Dove, 2000). Rabbits are easily milked and the milk naturally contains 2.5 times as much protein as sheep milk and 4.8 times that of goat milk (Jennes, 1974). Assuming 1 g/l mature protein expression, it has been estimated that as few as 54 transgenic rabbits could supply the entire U.S. market with rhFVIII (Wall *et al.*, 1997). Mammary gland-specific hFVIII gene constructs have previously been expressed in transgenic pigs (Paleyanda *et al.*, 1997) and sheep (Niemann *et al.*, 1999) with mixed success. Here we report, for the first time, the generation of transgenic rabbits bearing a rhFVIII transgene, analysis of rhFVIII mRNA and protein expression, and measurement of the biologic activity of the recombinant product.

MATERIAL AND METHODS

Generation of transgenic rabbits

The construct mWAP-hFVIII-Mt-I contains the 2.4-kb Eco RI-Kpn I fragment of the murine whey acidic protein (WAP) promoter from plasmid pP$_2$5'WAP (a gift from L. Hennighausen) ligated with the 9-kb Cla I-Sal I fragment of pSP73E plasmid that harbors the human Factor VIII cDNA, murine metallothionein (MT-I) introns and the SV40 poly(A) signal (Espanion *et al.*, 1997). Efforts to introduce the mWAP-hFVIII-MT-I into ovine zygotes failed to produce any transgenic offspring. For microinjection, the insert was cut from its plasmid by Pvu I–Sal I digestion, separated by electrophoresis on

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an 0.8% agarose gel in TAE buffer and finally recovered from the gel and purified with an ELUTIP-D NA010/0 (Schleicher & Schuell GmbH, Dassel, Germany). Collection of rabbit zygotes, microinjection, and transfer of injected embryos were performed as described (Besenfelder *et al.*, 1998).

Determination of transgene integration

Offspring derived from recipient does were screened for transgene integration by southern blot analysis as follows: 10 μg of genomic DNA purified from ear punch tissue or blood were digested with 5 units Bam HI at 37°C overnight. The restriction fragments were separated on a 0.8% agarose gel in TAE and blotted overnight onto Hybond N⁻ nylon membranes (Amersham AP Hungary Kft., Budapest, Hungary). A 9-kb Cla I–Sal I fragment of the gene construct was ^{32}P-labeled using the Hexalabel DNA labeling kit (MBI Fermentas, Biocenter Ltd., Szeged, Hungary) and hybridized to the blots. Autoradiographs were produced by exposure of the blots to Hyperfilm MP (Amersham AP Hungary Kft., Budapest, Hungary) overnight at −70°C.

Digestion with the restriction enzyme Bam HI followed by Southern blotting was used to determine the number of integrated copies of the transgene. This demonstrated single-copy integration but also revealed the loss of a Bam HI restriction site in the 3′ end of the integrated construct. To determine the extent of deletion, the 3′ end of the integrated construct together with flanking DNA was sequenced (Does *et al.*, 1991). Briefly, genomic DNA was digested with Hae III, and the fragments were purified and incubated with T4 ligase overnight to produce circular DNA fragments that served as templates for nested PCR with primers designed to hybridize back to back in the 3′ sequence so that the entire circularized target fragment was amplified.

Primers:

Primer 1(nt 2600-2624, Genbank ac.no.: J02400)
5′—CATCACAAATTTCACAAATAAAGCA—3′
Primer 2 (nt 1508–1527, Genbank ac. no. V00835)
5′—AGCTTGGCTTTACCCAAAGA—3′
Primer 3 (nt 2652–2676, Genbank ac. no. J02400)
5′—TTGTTGTTAACTTGTTTATTGCAGC—3′
Primer 4 (nt 2740–2763, Genbank ac. no. J02400)
5′—AAACCTCTACAAATGTGGTATGGC—3′

The two nested PCR reactions were based on 35 cycles of 95°C for 30 sec, 61°C for 45 sec, and 72°C for 1.5 min. Primers 1 and 2 served as outer primers, and primers 3 and 4 were the inner primers. An aliquot of 2 μl from the first reaction was used as template for the second reaction. Amplified products were resolved on 2% agarose gels, purified and sequenced.

Analysis of rhFVIII mRNA expression by RT-PCR

Mammary gland tissue biopsies were obtained from homozygous lactating females of line 385 between the first and fourth week of lactation. One lactating female was killed in the fourth week of lactation, and tissue samples were collected from brain, heart, liver, spleen, kidney, and salivary gland to test for ectopic expression. For RT-PCR analysis poly(A)⁺RNA was isolated using the Qligotex mRNA mini kit (Qiagen, Kasztel-Med Ltd., Budapest, Hungary). Following reverse transcription, the synthesized cDNA served as a template for PCR amplification using hFVIII-specific primers RH3 and RH4, which produce a 334-bp product (Espanion *et al.*, 1997). The RT-PCR reaction was carried out with a GeneAmp RNA PCR kit (Applied Biosystems, Budapest, Hungary) using 34 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. Amplified products were resolved on 2% agarose gels.

rhFVIII protein expression analysis by Western blotting

To remove fat, 50 μL milk samples were diluted by addition of 200 μL Milli-Q water and centrifuged at 800 × *g* for 30 min. Then 50 μL aliquots were further diluted with 50 μl of the 2× $NaDodSO_4$-PAGE sample buffer and boiled for 5 min. Finally, 10 μl of each sample was loaded onto an $NaDodSO_4$-PAGE gradient gel. After blotting onto a ProBlot membrane (Applied Biosystems), rhFVIII specific bands were detected with a primary polyclonal goat antibody against hFVIII (produced at the Institute for Animal Science), which was then visualized with a peroxidase conjugated F(ab′)2 fragment of rabbit antigoat IgG (H+L) (Cat. #305-036-045, Jackson Immuno Research Laboratories, Inc., West Grove, PA) followed by ECL chemiluminescent detection (Amersham AP Hungary Kft.).

Determination of clotting activity in rabbit milk

Milk samples were collected from the homozygous females twice a week over 4 weeks postpartum. Mothers were separated from their pups overnight, injected with oxytocin (0.5 IU), then anesthetized and milked. The clotting activity of milk samples was determined with an Immunochrom FVIII:C kit (Immuno GmbH, Heidelberg, Germany). This assay allows chromogenic determination of hFVIII activity, and is based on formation of a complex between FVIII, FIX, phospholipid, and calcium. This complex, in conjunction with FX, causes release of *p*-nitroaniline from an artificial substrate, which can be measured by its absorbance at 405 nm (Niemann *et al.*, 1999). Samples are compared to four standards supplied with the kit containing 1.30 IU, 0.7 IU, 0.05 IU, or 0.005 IU of hFVIII, calibrated against the WHO plasma standards. One IU is defined as the amount of hFVIII activity in 1 ml of normal human plasma.

RESULTS

Generation of transgenic rabbits

In total, 627 microinjected zygotes were transferred to 29 recipient does, and 14 females delivered 51 pups, among which three transgenic founder animals were identified (Hiripi *et al.*, 2000). Founder 385 was a male, which transmitted the transgene after mating to four does. The remaining two founders died due to causes unrelated to the experiment before offspring could be obtained. Southern blot analysis revealed that founder 385 had a single integrated copy of the transgene but a Bam HI site was missing at the 3′ end. Sequencing of this region showed that the terminal 23 bp of the injected construct were missing. This is nontranscribed DNA downstream from the SV40 poly(A) signal sequence and is not involved in transgene expression. Eight of the 31 pups (26%) produced by the first mat-

ing carried the transgene, suggesting a degree of mosaicism in the founder animal. Consecutive brother–sister matings established a homozygous line as confirmed by both Southern analysis (Fig. 1: lanes 3, 4, and 5) and test matings with nontransgenic rabbits.

Expression of FVIII in the mammary gland of transgenic rabbits

In biopsied mammary gland tissue from a female transgenic rabbit, rhFVIII mRNA was demonstrated by RT-PCR as a 334-bp product. Sequencing confirmed 100% homology with the published sequence for hFVIII. Except for the first week of lactation, rhFVIII mRNA was identified throughout the lactation period, indicating a delay in transgene induction (Fig. 2: lanes 2, 3, 4, and 5). Recombinant hFVIII mRNA was restricted to the mammary gland in homozygous females except for weak expression in the liver (data not shown).

In milk samples from homozygous lactating females, a rhFVIII-specific band of 190 kDa was detected by Western analysis, which was never found in negative controls. The strongest signals were observed during mid-lactation (Fig. 3: lanes 3 and 4). This 190-kDa fragment originates from proteolytic processing at position 740/741 instead of 1648/1649 (Pitmann and Kaufman, 1989) and explains the absence of the 80- and 73-kDa fragments, which would be expected in the case of normal processing of the 265-kDa precursor. The same 190-kDa fragment was observed in transgenic sheep bearing a β-lac-hFVIII-MT-I construct (Niemann *et al.*, 1999).

Functional activity of rhFVIII in milk

The clotting activity in milk samples varied between animals and over the course of lactation in the homozygous line. Clotting activity was barely detectable in the first 3 weeks of lactation but in the fourth week of lactation, the mean of two measurements on each of three homozygous females was 0.067 IU/ml (6.7% the level of normal human plasma) and the range was 0.052 to 0.083 IU/ml.

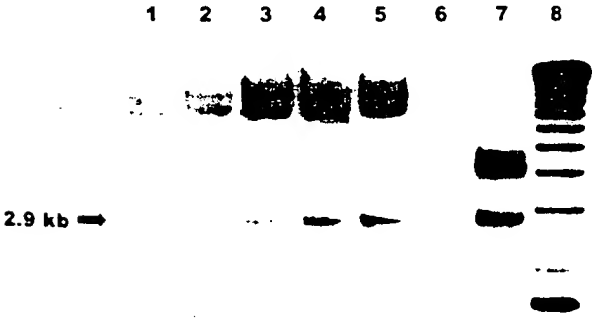

FIG. 1. Southern analysis of rhFVIII expressing rabbits. Lane 1: no. 103 heterozygous; lane 2: no. 126 heterozygous; lane 3: no. 161 homozygous; lane 4: no. 164 homozygous; lane 5: no. 168 homozygous; lane 6: nontransgenic control; lane 7: injected DNA (1 μL); lane 8: 1 kb DNA ladder (Gibco).

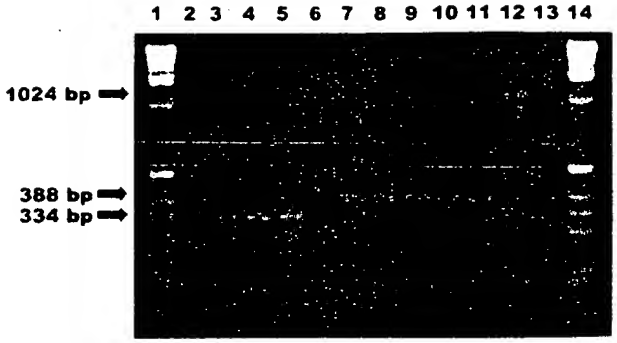

FIG. 2. RT-PCR analysis of Poly(A)$^+$RNA from mammary gland tissue biopsies of homozygous transgenic rabbit. Lanes 1 and 14: 1-kb DNA ladder (Gibco), lanes 2–5: RT-PCR of the mammary gland biopsies (no. 161/1) from the first to the fourth weeks of lactation, using the RH3 and RH4 primers (Espanion *et al.*, 1997), lane 6: RT-PCR of a nontransgenic negative control using the RH3 and RH4 primers, lanes 7–10: RT-PCR of the mammary gland on the same samples as in lanes 2–5 using α-actin primers (nt 917–938 and nt 1277–1299 GenBank ac no: X60732), lane 11: RT-PCR of a nontransgenic negative control using α-actin primers, lanes 12–13: PCR of genomic DNA from transgenic rabbit no. 161/1024 bp PCR product (Niemann *et al.*, 1999), and from nontransgenic rabbit respectively, using the RH3 and RH4 primers.

DISCUSSION

The rationale behind creating transgenic rabbits with mWAP-hFVIII-Mt-I was that the transgenic rabbit is a very efficient system for testing new gene constructs. The rabbit reproductive cycle is much shorter than that of pigs or sheep, and

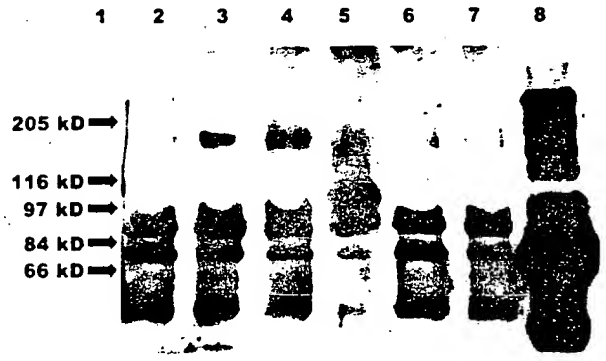

FIG. 3. Western blot analysis of homozygous transgenic rabbit milk samples. Note the specific band at 190 kDa, which originates from proteolytic processing at position 740/741 instead of 1648/1649 (Pittman and Kaufman, 1989). Ten microliters of 10× diluted milk samples were loaded. Lane 1: molecular weight marker; lanes 2–5: rabbit (no. 161/1) milk samples collected from the first to the fourth weeks of lactation; lanes 6–7 nontransgenic rabbit milk samples; lane 8: 10 μL of the hFVIII activity calibration standard provided with the Immunochrom FVIII:C reagent kit diluted to 0.001 IU/ml. [This was not a concentration standard, and was used only as a positive control for the immunochemical reaction and to demonstrate the location of hFVIII fragments on the blot.]

lactating rabbits produce more milk than mice (Van der Hout *et al.*, 2001). We have performed functional (i.e., clotting) tests with hFVIII in transgenic mouse milk (Espanion *et al.*, 1997) but the 50 ml of milk available per day from rabbits is an advantage in light of the low levels of expression achieved to date. The increased amount of hFVIII obtained in rabbit milk will facilitate the investigation of glycosylation differences related to the rabbit mammary gland and the effect on activity and immunogenicity. Functional tests are necessary for proteins expressed in transgenic animals because of species differences in glycosylation (Raju *et al.*, 2000) and alternative splicing (Aigner *et al.*, 1999).

The special considerations in factor VIII production are its large size, and the need for glycosylation, endoproteolytic processing, and modification by a vitamin K-dependent carboxylase that converts selected aminoterminal glutamic acid residues to γ-carboxyglutamic acid. Recent studies of species specificity in the glycosylation of proteins such as IgG underscore the significance of selecting the appropriate species for hFVIII production (Raju *et al.*, 2000). The rate limitation of the γ-carboxylation of human protein C in the mammary glands of transgenic mice and pigs suggests that it is important to perform similar tests with recombinant proteins produced in rabbit milk (Subramanian *et al.*, 1996). Species-specific differences in alternative splicing were observed when the genomic sequence for human growth hormone was used for production of transgenic rabbits and pigs (Aigner *et al.*, 1999). Although this is a characteristic of the growth hormone gene, it is not clear that it is a general phenomenon. It is possible that alternative splicing will be a problem when constructs based on the genomic hFVIII sequence are used. Alternative splicing is an important feature of the growth hormone gene, but has not been reported for the FVIII gene except where a splice site mutation resulted in exon skipping causing hemophilia.

After submission of this manuscript, it was reported that a higher level of expression of FVIII cDNA has been achieved in transgenic mice using the bovine α-lactalbumin promoter (Chen *et al.*, 2002) than has been possible with either the bovine β-lactoglobulin promoter (Niemann *et al.*, 1999) or the murine whey acidic protein promoter (Paleyanda *et al.*, 1997) employed in transgenic sheep and pigs, respectively. It is clearly important to test a similar construct in the rabbit and eventually in a larger animal.

From this study, it is clear that the murine WAP promoter gives more stringent control of rhFVIII expression specific to rabbit mammary gland than the ovine β-lactoglobulin promoter had been in transgenic sheep (Niemann *et al.*, 1999). This fact is underlined by a report that the murine WAP promoter gives mammary gland-specific expression in transgenic rabbits with ectopic expression restricted to one or two other tissues (Castro *et al.*, 1999).

Although the expression levels currently obtained with the mWAP-hFVIII-Mt-I construct have not been sufficient for analysis of post-translational modifications, it is anticipated that further improvements in the transgene construct (e.g., use of a modified cDNA sequence hVIII or the use of the genomic sequence for hFVIII under the control of mammary gland specific promoter) will lead to increased expression levels in the future. The rabbit provides an excellent basis for making rapid progress in this field, as it is possible to obtain homozygous animals only 12 months after the birth of a transgenic founder and lactating homozygous animals 6 months later.

In summary, for the first time, biologically active rhFVIII has been produced in the mammary gland of a transgenic rabbit where the mWAP-hFVIII-MT-I construct gave stronger expression than previously achieved with β-lac-hFVIII-MT-I in sheep.

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